THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 1/17/74

1. Principal Investigator (give title and degrees):
Herbert McKennis, Jr., Ph.D.
Professor of Pharmacology

2. Institution & address:
Department of Pharmacology
Medical College of Virginia
Richmond, Virginia 23298

3. Department(s) where research will be done or collaboration provided:
Department of Pharmacology, Medical College of Virginia
Department of Chemistry, Duke University
Department of Toxicology, Karolinska Institutet, Stockholm, Sweden

4. Short title of study:

Pharmacodynamics of Cotinine

5. Proposed starting date: April 1, 1974

6. Estimated time to complete: Three years (with publishable results within first year).

7. Brief description of specific research aims:

The specific research aims on the pharmacodynamics of cotinine, a principal metabolite of nicotine, are directed toward an understanding of the possible contribution and role of cotinine and its metabolites in physiological responses to nicotine. Since the list of metabolites is lengthy and real or alleged responses to smoking are numerous, it is the intent during the first year to emphasize only those aspects of the problem in which there are already well-established, interesting leads. These principle current leads arise primarily from past studies here and elsewhere on cardiovascular and lipid-metabolism effects and suggest that some of the metabolites of cotinine may serve to block through direct competition the action of nicotine at various receptor sites.

8. Brief statement of working hypothesis:

The metabolism of nicotine via cotinine leads to a number of compounds in which there has been further alteration or degradation of the pyrrolidine ring of the parent nicotine. These transformations take place at varying rates depending upon genetic control, dietary and other factors which affect pH of physiological compartments and structures, rate of absorption, excretion, etc. This permits possible participation of numerous nicotine metabolites in various physiological functions and in the possible suppresion of various effects attributed to nicotine by itself. The extent to which these effects may be observed will be dependent in part upon rates of formation and on degree of concentration of the metabolites in various parts of the body. Various lines of evidence suggest that cotinine, which is detectable in the body long after nicotine has disappeared, may participate in producing some subtle physiological consequences which may become important as a result of future study.

After the initial isolation of cotinine as a mammalian metabolite of nicotine (McKennis, Turnbull, and Bowman, J. Amer. Chem. Soc. 79, 1342 (1957)), it was established that rather large quantities of cotinine administered to man and other animals under varying conditions produced

(continued)

9. Details of experimental design and procedures (append extra pages as necessary)

(see page 4)

8. Brief statement of working hypothesis:

Continued...

no apparent adverse reactions. For example, in one experiment (McKennis, Turnbull and Bowman, J. Biol. Chem. 238, 720 (1963)) a male subject received 10.8 g of cotinine individed doses over a six day period for the purpose of obtaining nicotine metabolites that are derived from the intermediate cotinine. No adverse effects were reported, and in another study lesser amounts of cotinine (approximately 50-100 mg per day orally for several weeks) were received orally by 33 subjects. The clinical reports are of such a nature as to suggest that cotinine is well-tolerated.

Recent data from various laboratories show a rapid disappearance of nicotine from the blood of human subjects following smoking (see, for example, Langone, Gjoka, and Van Vunakis, Biochemistry, 12, 5025 (1973)). The rapid fall in nicotine levels in the blood is followed by a rise in the reported cotinine levels in the serum. Cotinine is then excreted in the urine as itself or in the form of a variety of mammalian metabolites which include many in which there has been further chemical alteration of the pyrrolidine ring of the parent nicotine. The list of compounds thus implicated in the nicotine metabolism, which is rather extensive and has been previously reported in the literature and in reports from us to the Council for Tobacco Research, includes 3-pyridylacetic acid, 4-3-pyridyl-4-oxobutyric acid, 4-3-pyridyl-3-methylaminobutyric acid, 4-3-pyridyl-4-hydroxybutyric acid, 4-3-pyridyl-3-butenoic acid, 4-3-pyridylbutyric acid, N-3-pyridylacetylglycine, 3-hydroxycotinine, 5-hydroxycotinine, and demethylcotinine.

In essence, one may say that cotinine, which is clearly detectable in the blood many days after the cessation of smoking, represents a slow release form (via metabolism) of the listed substances.

9. Details of experimental design and procedures:

For illustration, provisional experimental designs and procedures are briefly summarized in three areas in which some of the interesting results or leads already have been obtained.

I. Effect of Cotinine Metabolites on Lipid Metabolism

Various experimental procedures that have been employed in man to study the effect of numerous agents in depressing or elevating serum free fatty acid (FFA) levels have been described. In particular those related to smoking have been reviewed in the Tobacco monographs of Larson et al. Other aspects are reveiwed in Metabolic Effects of Nicotinic Acid and Derivatives, edited by Guy and Carlson, Hans Huber Publishers (1971). Some of the difficulties of such studies include stress or excitation to the subjects as a result of the experimental procedures. The stress may often mask the inherent activity of compounds under physiological investigation.

Recent comparative studies on the effects of various 3-pyridyl-alkyl carboxylic acids on preventing epinephrine-induced FFA rise in dogs. It becomes desirable to parallel the techniques described in the two cited by Carlson et al in initial experiments. It may be noted that there already exists data (Acta Pharm. Suecica 9, 405 (1972)) attributing to 4-3-pyridyl-butyric acid on a molar basis a greater potency in suppressing FFA rise than that of 3-pyridylacetic acid. Both of these acids are involved in the metabolism of nicotoine. By analogy with other data, 4-3-pyridyl-3-butenoic acid and 4-3-pyridyl-4-hydroxybutyric acid may have similar interesting action in this regard. These substances, N-3-pyridylacetyl-glycine, and other substances in the degradation of cotinine deserve experimental consideration, since cotinine may be considered to be a slow-release form of the various compounds. Additionally, since 4-3-pyridylbutyric acid is probably metabolized to 3-pyridylacetic acid, the butyric acid derivative and other nicotine metabolites can be considered slow release forms of 3-pyridylacetate.

II. Inhibition of Direct and of Indirect Effects of Nicotine on Smooth Muscle

Various model systems of varying degress of simplicity have already been employed in this laboratory to search for possible inhibitory control of the effects of nicotine on smooth muscle systems. The procedures which are based on whole or in part in application of standard literature to various pyridyl compounds and nicotine metabolites (Kim, Borzelleca, Bowman and McKennis, J. Pharm. Exp. Therap. 161, 59 (1968); Konzett, Bost, Bowman, Bowman, and McKennis, J. Pharm. Exp. Therap. 178 122 (1971); McKennis, Chang, Bowman, and Wilson, Fed. Proc. (1974) submitted recently and previously supplied by copy to the Council).

(a) Aortic Strips - A possible suggestion of validity already established for the experimental design arises from studies (recently completed and unpublished) on contraction of rabbit aortic strips. After first establishing a standard response to nicotine and metanicotine, it was shown that pretreatment with two selected nicotine metabolites 3-pyridylacetic acid and N-3-pyridylglycine provide a partial or total block, inhibition of the contraction produced by nicotine or metanicotine. Precise mathematical relationships between the dosages required for stimulation and blockade with the various substances remain to be determined.

(b) Isolated Intestinal Segments - Practicality of the use of isolated intestinal segments for studies in vitro on possible antagonism between nicotine metabolites and nicotine itself has been previously demonstrated (Kim, Borzelleca, Bowman and McKennis, J. Pharm. Exp. Therap. 161, 59 (1968). In this early study it was shown that cotinine methonium ion blocked the response to nicotine, but not acetylcholine, barium chloride, or histamine, in segments obtained from the rabbit.

(c) Peripheral Vascular Resistance in the Perfused Forelimb of the Dog

This model has been employed (Konzett et al, J. Pharm. Exp. Therap., 178, 122 (1971) in this laboratory to study the histamine-like effects of betahistine during the course of clinical work by other investigators, who noted improved circulation in the brain and other organs (excluding the kidney) as a result of oral administration of the compound. The use of this preparation for studies on nicotine and its metabolites has already been reported to the Council for Tobacco Research - USA. With some substances in the nicotine series a "trimodal" type of distribution in biological response was noted. It has been suggested to us that a definable genetic basis is implied by the type of distribution. Irrespective of interpretation, this type of variation has limited the usefulness of the prepration in many studies. However, the simplicity of the surgical procedure to provide blood delivery at constant flow with changes in resistance (pressure) readily measured makes the preparation useful adjunct in experimental studies.

(d) Possible Mechanism of Physiological and Biochemical Antagonism to Some of the Actions of Nicotine

Preliminary studies on the effect of diamine oxidase (pig kidney origin) on various nicotine metabolites were reported in a report to the Council (October 1, 1973). The data suggest that dihydrometanicotine and metanicotine, two metabolites of nicotine, are rapidly oxidized by the enzyme preparation and that these substances can inhibit histamine oxidation. Such studies are of interest if one considers that contraction of capillary sphincters is under adrenergic control and that this contraction is opposed by histamine. In other words some instances of improved or unimpaired microcirculation following exposure to nicotine may be ascribable to a biochemical protection of local histamine which may be capable of opposing the actions of local or peripherally released adrenergic substances. Other interpretations, including inducement of platelet deaggregation, are of course possible considerations. Enzymatic studies of this type and others have, of course, a direct and indirect bearing on central nervous system effects of nicotine.

(e) Chemical Considerations

In the sequence of known compounds leading from nicotine through cotinine to 3-pyridylacetate and its glycine conjugate, which is more potent than pyridylacetate as a nicotine antagonist in preliminary studies in vitro, methods for synthesis have already been described in publications from this laboratory and are cited as references or additional references in section 13. The most recently reported and most convenient route to 5-hydroxycotinine is via dibromoticonine. The method for synthesis of the latter appears in an attached reprint (J. Chem. Soc., Perkins Transactions I, 2046-2049 (1973)).

(f) General Comments

On the basis of preliminary studies already conducted, it is reasonable to anticipate an increasing number of instances in which pharmacological antagonism to nicotine by its metabolites may be uncovered. Since better assay methods for the metabolites of nicotine and nicotine itself are continually being developed throughout the world, it will become increasingly easier to interpret the possible significance of these pharmacological events. Already it has been demonstrated from the experiments of Schmiterlöw et al and of Waddell that there is considerable difference in the distribution of the radioactivity of nicotine-C^{14}-methyl (after injection of the compound into various strains in mice. This difference may reflect a difference of concentration of nicotine or metabolites in various areas of the body; such features may suggest a pharmacological activity of some metabolites as "anti-nicotines" in excess of that activity now projected from smoking data and existing data on cotinine levels in plasma which follow smoking of tobacco.

Although not part of this study, there are additional features which make the study interesting to us. These include the possibility that some seemingly desirable central effects of nicotine may be mediated in whole or in part by peripheral effects. As an example, it has been considered that some of the desirable psychological consequences of cotinine reported by human subjects could be ascribed to a mild muscular relaxant property.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Two laboratories (approximate total of 800 sq. ft.), well-equipped for chemical and pharmacological studies, are available for these studies. In addition, there are two instrument rooms which house spectrographic, chromatographic, and radioactive counting equipment. Animal quarters (shared with others) are available for small mammals and large animals (horses, etc.) are kept in rented areas or at a school animal farm.

List of some major items of permanent equipment available for this work:

Cary recording spectrophotometer, model 11-PM
Grass polygraph, six channel, model 5
Nuclear-Chicago liquid scintillation system, 720 series
Beckman amino acid analyzer, model 120B
Perkin-Elmer gas chromatograph, model 801
Nuclear Chicago gas chromatography counting system
Wilkens Aerograph Autoprep, model A-700
Preiser Scientific integrator-printer
Wilkens Aerograph 200 (2 each)
Nuclear-Chicago Actigraph III paper radio chromatography system
International preparative ultra centrifuge, model B-35
Vacuum pumps (six of various types)
Warburg Apparatus
Hewlett Packard Model 5700A Gas Chromatograph with integrator
Chemical balances (4 each)
Zeiss photoelectric polarimeter
Cahn electrobalance

(continued)

11. Additional facilities required:

If any, this would be determined by the outcome of the investigations.

12. Biographical sketches of investigator(s) and other professional personnel (append):

(see page 8)

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

10. Space and facilities available:

Continued...

Fraction collectors (2 each)
Miscellaneous glass metabolism cages, distillation equipment, chromatography equipment
Radiometer pH meter, O_2CO_2 determinator
Blood oxygenator (local design for organ perfusion)
Varian A-60 NMR apparatus
DuPont Model 830 Liquid Chromatography Apparatus

12. Biographical sketches of investigator(s) and other professional personnel:

NOTE: These are appended for principal investigator and co-workers, who are experienced in areas included in proposed study. Those not included in 14A (salaries for the first year budget) may be attracted on a limited voluntary basis and a more extensive basis if funding can be later accomplished through sources which have not presented themselves or been solicited. (No formal or informal request has been made to other possible sources.)

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Herbert McKennis, Jr., Ph.D. (Professor)	20%	$ -----
Edward R. Bowman, Ph.D. (Research Associate)	100%	$22,846.00
Faye J. Bowman, Ph.D. (Research Associate)	70%	$10,773.00
Arthur W. Burke, Jr., M.D., Ph.D. (Resident in Radiology)	Undetermined	
Technical		
Kendall L. Wilson, Jr., M.S. (Lab Specialist)	Undetermined	

Sub-Total for A $33,619.00

B. Consumable supplies (by major categories)

(FUNDS FOR CATEGORIES B, C, AND D TO BE SOUGHT ELSEWHERE)

Sub-Total for B _____

C. Other expenses (itemize)

Sub-Total for C _____

Running Total of A + B + C $33,619.00

D. Permanent equipment (itemize)

Sub-Total for D _____

E. Indirect costs (15% of A+B+C) $ 5,042.85

Total request $38,661.85

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$35,299.95				$5,295.00	$40,594.94
Year 3	$37,053.41				$5,558.02	$42,611.43

10035540329

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Factors Controlling the Development of Pharmacologically Active Derivatives of Nicotine	American Medical Assoc.	$45,750.	7/1/73 - 6/30/74
Biological Activity of Tobacco Smoke Components and Allied Substances	Council for Tobacco Research - USA #868	$30,000.	10/1/73 - 4/1/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Dr. Herbert McKennis, Jr.

Signature Herbert McKennis, Jr. Date 1/17/74

Telephone (804) 770-4406
Area Code Number Extension

Checks payable to

lter P. Lossing, Comptroller-Treasurer

Mailing address for checks
VCU/MCV
1200 East Broad Street, Richmond, Va 23298

Responsible officer of institution

Typed Name M. Pinson Neal, Jr., M. D.

Title Provost VCU/MCV

Signature [signature] Date

Telephone (804) 770-5150
Area Code Number Extension

10035403330